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Article

Use of Native Promoter-eGFP as a Gene Reporter on Onion Epidermis to Analyze Gene Expression of *AVR-Pia*, an Avirulence Effector of Rice Blast Pathogen

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Abstract. Rice blast disease, caused by *Magnaporthe oryzae*, is a major rice disease over the world. Recent studies have identified avirulence effectors from the blast fungus that trigger rice immune against the pathogen invasion after specific interaction with resistance (R) proteins in rice. AVR-Pia is one of avirulence effectors that correspond to Pia-resistant protein, inducing hypersensitive response (HR). Enhanced Green fluorescent protein (eGFP) was used as a reporter of *AVR-Pia* expression in this study. We synthesized expression vector containing native promoter of *AVR-Pia* (*PRR*) fused to *eGFP* gene. Rice sheath assay was done to observe the fluorescence and the signal was found in appressoria and invasive hypha of *M. oryzae*, suggesting that *AVR-Pia* is expressed in appressorium and invasive hyphae after penetration. Although, rice sheath assay is a reliable way to study rice-pathogen interaction, it is a consuming-time method. Onion epidermis was tested to check the availability to use as model system instead of rice sheath. After inoculation *M. oryzae* containing *PPR::eGFP* on onion epidermis, fluorescence was observed in appressoria and invasive hypha of transformants similar to observation on rice sheath. Therefore, onion epidermis can be used as plant cell model to study *M. oryzae* effectors expression by fluorescence observation.

Keywords: *Magnaporthe oryzae*, *AVR-Pia*, *PPR*, *eGFP*, onion epidermis.

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1. Introduction

Rice disease is one of the major causes that limit the increase of rice production to meet the expected rice demand due to ever-expanding human population. Among rice diseases, rice blast is considered as serious and widespread disease in all rice-growing regions. Pathogen causing rice blast is an ascomycete fungus, *Magnaporthe oryzae*. This fungus can attack all the aerial parts of the rice such as leaf, node, neck, and panicle. The conidia of *M. oryzae* have to attach to the rice leaf as soon as possible because it cannot be infective in a long period without attachment. Rice surface is covered with waxy-cuticle, which makes them become hydrophobic. Once attached, the conidium germinates and develops a short germ tube. After several hours, germ tube stops growing at the tip and a 'hooking' of the hypha appears, preparing for the beginning of differentiation of an appressorium that is required for infection of the plant. Appressorium is in a dome shape with thick cell wall, which contains of outer fibrillar layer and inner layer of melanin [1]. The appressorium plays an important role in facilitating tightly adhesion and penetration. Accumulation of glycerol in the appressorium then gives turgor pressure that empowers the fungal penetration. After 20 h, penetration peg is formed at the base of appressorium and penetrate into rice cell, facilitated by such appressorial turgor pressure. After localization of the fungus thoroughly in host cell, the fungus produces more conidia on the lesion, and then releases many new airborne spores to infect other rice plants and restart the cycle again. Rice blast infection requires favourable environmental conditions such as long periods of plant surface wetness, high humidity, little or no wind at night and night temperatures between 12 – 32 °C [2].

In order to control rice blast diseases, the most effective and environmentally friendly approach is breeding and planting resistant cultivars. Thereby, understanding of plant immunity is necessary to find the way to make the durable resistant cultivars as a novel agricultural application. Plant immune system is currently understood to consist of two layers [3]. First layer is called pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI), governed by transmembrane receptors, or pattern-recognition receptors (PRRs). PRRs recognize conserved PAMPs, inducing a relatively weak immune response that inhibits colonization of pathogens. The second layer, Effector-triggered immunity (ETI) is a rapid and robust response and based on highly polymorphic resistance (R) proteins that are activated by the recognition of corresponding pathogen molecules called avirulence (AVR) effectors. This interaction has become known as 'gene-for-gene' hypothesis [4]. ETI associates with hypersensitive response (HR), a response consisting in host cell necrosis at the site of infection [5].

Since avirulence effectors can efficiently prevent the pathogen infection as mentioned, recently many researchers have been interested in AVR effectors and expect that the information of AVR effector enables us to understand the mechanisms and helps the humanity to succeed in fungal pathogen regulation. To date, ~85 blast R genes have been identified [6]. In Japanese rice cultivars, thirteen dominant genes (*Pish*, *Pik-h*, *Pia*, *Pii*, *Pik*, *Pik-m*, *Piz*, *Pita*, *Pita-2*, *Piz-t*, *Pik-p*, *Pib*, and *Pit*) have been identified, while twenty –five avirulence genes of *M. oryzae* have been genetically mapped [7], nine of which were recently isolated, *AVR-Pita* [8], *AVR-CO39* [9], *PWL1* [10], *PWL2* [11], *ACE1* [12], *AVR-Pizt* [13], *AVR-Pia* [14], [15], *AVR-Pii*, and *AVR-Pik/km/kp* [16]. The avirulence genes *PWL1* and *PWL2*, isolated by map-based cloning are responsible for the nonpathogenicity of rice pathogenic strains of *M. oryzae* against *Eragrostis curvula*, weeping lovegrass [10], [11]. *AVR-Pita* gene was also isolated by map-based cloning. This AVR effector is related in resistance to rice cultivars harboring the *Pita*-R gene [8]. *ACE1* encodes a hybrid polyketide synthase/ nonribosomal peptide synthetase (PKS-NRPS) probably involved in the biosynthesis of a secondary metabolite, recognized by *Pi33* resistant gene [17]. AVR-Pia is one of avirulence effectors that be recognized by the rice Pia-resistance protein via gene-for-gene relationship. *AVR-Pia* was first revealed by comparison of Ina168 and its spontaneous *avr-Pia* mutant, *Ina168m95-1* [14]. *AVR-Pia* is 255 bp in length, containing a 57 bp of secretion signal.

In this study, we monitored *AVR-Pia* expression using green fluorescence protein (*GFP*) gene as gene reporter. Gene expression is a crucial step to control the production of any proteins from the earliest molecules, called deoxyribonucleic acids (DNAs). Gene expression processes are composed of transcription and translation, which produce ribonucleic acids (RNAs) and proteins respectively. In order to study gene expression, a powerful method is using green fluorescence protein (GFP) as a marker of gene expression. *GFP* gene was first used to produce strong fluorescence light in prokaryotic and eukaryotic cells in 1994 by Martin Chalfie [18]. However, wild-type *GFP* is not efficiently applicable to many fungi because of poor translation. Many versions of enhanced *GFP* have been made by chromophore mutation and codon usage optimization to be suited with different organisms such as plants (*SGFP*) [19], yeasts (*yEGFP*)

[20], and mammals (*EGFP1*) [21]. In 1999, *EGFP1* was successful expressed in *M. oryzae* to study calmodulin gene expression [22] and other filamentous fungi. Therefore, *eGFP* gene was chose in this experiment. Native promoter of *AVR-Pia* gene (*PPR*) was attached directly to *eGFP* gene to observe the regulation of *AVR-Pia* expression by *PPR*. To monitor the gene expression *in planta* during the rice blast infection, rice sheath assay is a finest way. Rice sheath assay is a method using sheaths of rice that have been removed intact from rice plants, designated as 'intact method'[23]. Infection process of *M. oryzae* was successfully observed in both susceptible and resistant rice plants. In this report, we used rice sheath assay to observe the *AVR-Pia* expression, controlled by *PPR::eGFP*. However, rice sheath assay is a time-consuming and requires a lot of nurtures. Onion epidermis is an alternative way for this study to shorten the experimental time because it does not take a lot of time to prepare the onion tissue. Transcriptional fusion between *PPR* and *eGFP* was used to test the availability of onion epidermis usage instead of rice sheath. In addition, hydrophobic glass slide was also used to observe morphology of *M. oryzae* during development and fluorescence expression. Normal appressoria were differentiated on glass slide and fluorescence was expressed in appressoria. Validity of these methods as alternatives for intact leaf sheath inoculation will be discussed in this paper.

2. Materials and Methods

2.1 Fungal Strains, Plant Materials and Vectors

The *M. oryzae* spontaneous mutant strain, Ina168m95-1 (*AVR-Pia* deletion mutant) was obtained from Miki S. [8]. Fungal isolate was stored at -20 °C as dried mycelium pieces of filter papers and cultured on oatmeal agar before use. Rice (*Oryzae sativa*) material used in this study was *Shin-2* rice, which is lacking of *Pia* resistant gene. Plasmid pCSN43 [24] was prepared to be ligated with a Rf-cassette (Gateway Vector Conversion Kit; Invitrogen) and became pCSN43-DEST, containing hygromycin phosphotransferase (hph) gene and gateway cassette [14]. *eGFP* was fused to native promoter of *AVR-Pia* (*PPR::eGFP*) and introduced into plasmid pCSN43-DEST. The resulting vector was called pCSN43-DEST-*PPR::eGFP* (Fig. 1). *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used for recombinant DNA experiments and vector construction.

2.2 Fungal Transformation Using Protoplasts

Fungal protoplasts of the recipient strain were prepared as described by [14] with some modifications. Ten days fungal cultures on oatmeal agar were scratched and keep incubated for more 3 days at 25 °C with fluorescent light. Conidia suspension was gained after filtrated mycelia suspension by 20 ml of 2YEG (2 g/L yeast extract, 10 g/L glucose). Conidia suspension was inoculated into 2YEG medium at 10^5 spores/ml and incubated at 27 °C for 3 days. Mycelia were harvested by filtering through miracloth (Calbiochem, San Diego, CA), and digested the cell wall by 20 mg/mL Yatalase (Ozeki, Nishinomiya, Japan) and 5 mg/mL Cellulase Onozuka (Yakult Honshya, Tokyo, Japan) with digestion buffer (0.6 M sucrose, 50 mM maleic acid monosodium salt, pH 5.5) at 37 °C with 1 hour shaking. The suspension was filtrated through miracloth (Calbiochem, San Diego, CA) in order to get the protoplast cells. Protoplast suspension was washed and stored in STC buffer (20% sucrose, 10mM Tris-HCl, pH 7.5, 50 mM $CaCl_2$). Transformation was done by PEG-mediated method. DNA vector was cut to be a linear strand by restriction enzyme and purified by standard phenol-chloroform extraction and ethanol precipitation method. Ten micrograms of DNA vector was added to 100 µl of protoplasts (10^8/mL) in a 50-mL conical tube, placed on ice for 20 min, then added 2 ml of polyethylene glycol (PEG) 4000 (60% Tris-HCl in 10 mM Tris-HCl pH 7.5, 50 mM Calcium chloride), mixed gently and kept on ice for 20 min. Then 30 ml STC buffer was added slowly, mixed gently and centrifuged at 3,500 rpm for 10 min at 4 °C. The supernatant was removed and the pellet was resuspended in 200 µl STC buffer. Twenty-five mL of Bottom agar (6 g/L yeast nitrogen base, 5 g/L glucose, 205 g/L sucrose, 1.5 % agar), warmed at 55 °C, was added into the pellet suspension. Mixed by inverting several times and aliquoted into two petri dishes, solidified and incubated at 27 °C overnight. After regeneration of protoplasts was confirmed by microscope, 12.5 mL of Top agar (as for bottom agar, but no sucrose, and added 800-1,000 µg/mL hygromycin B) at 55 °C was poured over the bottom agar layer, and incubated at 27 °C for 3-5 days. The colonies that could grow through top agar were transferred to oatmeal agar plates containing 400-500 µg/mL hygromycin B, and

incubated at 25 °C with light for 1 week. Single conidia was isolated by cutting a piece of each sample on oatmeal agar and stamped it over the water agar (40 % agar), then incubated at 27 °C overnight. Each germinated conidium was isolated using a needle under microscope and inoculated into 24 wells plate of prune agar (4 g/L prune extract, 1 g/L yeast extract, 5 g/L lactose, 5.56 % agar, containing 400-500 μg/mL hygromycin B), and incubated at 27 °C for 5 to 7 days.

2.3 Fungal Genomic DNA Extraction

After the putative transformants grew on 24 wells plate of HygB-prune agar, DNA of each isolate was extracted from mycelia to check the present of vector by polymerase chain reaction (PCR). Mycelia in each well of HygB-prune agar was incised to be a small piece of agar and putted into 1.5 ml microcentrifuge tube. Five hundred microliter of lysis buffer (50 mM KH_2PO_4, 50 mM K_2HPO_4, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% triton X-100, 10 mM imidazole) was added and the mycelia were ground. Then gDNA was precipitated by ethanol precipitation and DNA pellets were dissolved in 30 μl TE buffer. Genomic DNA of each sample was checked by PCR with designed primers. For southern hybridization, high quality of DNA extraction is necessary. Putative transformants were grown to form mycelia for 5 days with shaking in 40 mL of 2YEG at 27 °C. The mycelia were harvested and preapared for genomic DNA isolation using vacuum filtration technique and freezing by VD-500F freeze dryer (TAITEC, Tokyo, Japan). Freeze-dried mycelia were homogenized using multi-beads shocker (Yasui Kikai, Osaka, Japan). Five hundreds microliter of DNA extraction buffer (200 mM Tris-HCl pH 8.5, 25 mM EDTA pH 8.0, 250 mM NaCl, 0.5% SDS) was added to homogenized mycelia and mixed well. Then 350 μl of phenol and 150 μl of chloroform were added to mycelia suspension, mixed and centrifuged at 15,000 rpm for 1 h. The upper aqueous phase was pipetted into a new eppendorf tube, containing 25 μl of RNase and incubated at 37 °C for 1 h. After that, washed by 500 μl of phenol twice and washed again by 500 μl of chloroform. Isopropanol 0.6 volume of solution was used to precipitate DNA for 20 min and centrifuged at 15,000 rpm for 15 min. The pellet was washed with 70% ethanol and air-dried DNA pellets were dissolved in 50 μl TE buffer. High purity DNA samples were checked by southern hybridization with specific probe.

2.4 Intact Rice Sheath Assay

Conidia suspension of the transformant was prepared from 10-days-old oatmeal agar cultures. Conidia suspension was diluted to be 10^4-10^5 cells/mL. Shin-2 rice, lacking of *Pia* resistant gene, was used in this study. Seeds were sown in a plastic seedling pot containing soil with fertilizer (Honens seedling soil, Honen Agri, Co. Niigata, Japan), and grown in a greenhouse for 60 days. Intact leaf sheath inoculation was done as previously described [23] with some modifications. Leaf sheaths of the sixth leaves rice plant from outside of core leaf were peeled off until the last one nearest to the core leaf. The core leaf was drawn out to remain a leaf nearest the core leaf. The leaf sheath was laid horizontally on a support, and the conidia suspension was injected into leaf sheath using a syringe. The inoculated leaf sheaths were incubated at 25 °C for 24 and 48 h under 4000 lux of white fluorescent light a 16 h light period.

2.5 Glass Slide Inoculation and Onion Epidermis Assay

Onion epidermis has also been used as plant cell model in this study. Onions were cut to be 2 cm^2, and then peeled out the epidermis. The pieces of epidermis were heat-killed with a microwave oven for 45 sec in distilled water before dropping the conidia suspension (10^4-10^5 spores/ml), and incubate at 27 °C for 24h. Similar to onion epidermis, conidia suspension was inoculated on glass slide (Matsunami, Osaka, Japan) by dropping, and incubated at 27 °C for 24h.

2.6 Fluorescent Observation

Specimens for microscopy were prepared for fluorescent observation. Infected leaf sheaths were cut and the curve part of leaf sheath was sliced. The excised leaf sheath pieces and inoculated onion epidermis were put on slide prior to observe GFP signal using microscope. Microscopy was performed with a BX-50 fluorescent microscope (Olympus, Tokyo, Japan) equipped with an U-MNIBA3 filter set.

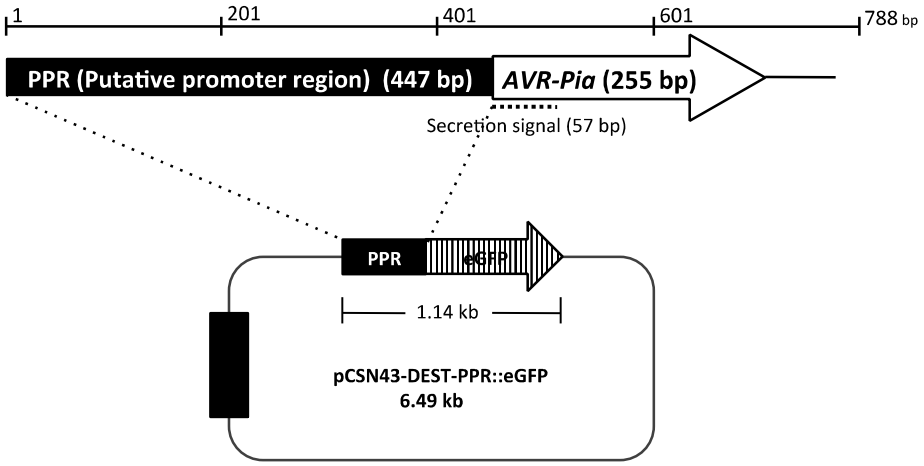

Fig. 1 Construction of expression vector pCSN43-DEST-*PPR*::*eGFP*. Promoter of *AVR-Pia* gene was attached to enhanced *GFP* gene. *Hygromycin-B* gene is a selectable gene for transformant.

3. Results

3.1 Native Promoter of *AVR-Pia* Fused with *eGFP* Gene Can Express Fluorescence *in vivo*

In order to study expression of *AVR-Pia*, 447 bp of native promoter of *AVR-Pia* (*PPR*) was fused to a reporter gene named enhanced green fluorescent gene (*eGFP*) and transformed to genomic DNA of *Ina168m95-1*, mutant of *M. oryzae* that lacks of *AVR-Pia* gene. Intact leaf sheath assay was used to observe the expression of *eGFP* gene by *PPR*. Conidia suspension of Ina168m95-1-*PPR*::*eGFP* was inoculated on 2 months-old leave sheath of *Shin-2* susceptible rice cultivar and incubated at 27 °C for 24 and 48 hours, and then observed fluorescent signal by fluorescent microscope. Ina168m95-1-*PPR*::*eGFP* developed the well-melanized appressoria at 24 hpi (hours post inoculation) on inoculated rice sheath (Fig. 2(a)). The expression of *GFP* was obviously seen in appressoria of *M. oryzae* (Fig. 2(b, c)). After 48 hpi, invasive hyphae were completely developed into rice cells, and it showed fluorescent signal in those invasive hyphae that colonized inside rice cells (Fig. 2(d)). The results indicate that *PPR* can regulate the expression of *eGFP* gene in appressoria and invasive hypha of *M. oryzae* (Fig. 2(e, f)). Moreover, it suggests that the expression vector, containing native promoter of *AVR-Pia* directly attached to *eGFP* gene, is successful to analysis *AVR-Pia* expression *in planta*.

3.2 Onion Epidermis Can be Used as Infection Assay for *Magnaporthe Oryzae*

Since intact rice sheath assay is time-consuming method and needs a lot of skills, other alternative ways are needed to shorten the time. We monitored *AVR-Pia* expression of Ina168m95-1-*PPR*::*eGFP* on glass slide and onion epidermis. Appressoria were differentiated normally on these membranes as similar as the observation on rice surface (Fig. 3(a, d)). Penetration of transformant was seen in onion cells by observing of invasive hypha (Fig. 3(g)), while no penetration occurred on glass slide. The difference of glass slide and onion epidermis is that no cell wall components are contained in glass slide. This result indicates that *M. oryzae* is able to penetrate into onion cells. In addition, fluorescence signal was found in maturated appressoria on glass slide and onion epidermis after 24 hpi (Fig. 3(b, e)). Moreover, fluorescence was also seen in invasive hypha, which colonized into onion cells after 24 hpi (Fig. 3(h)). These results showed that AVR-Pia expression is induced both in appressoria on glass slide and onion epidermis, and after penetration the expression was found in invasive hypha on onion epidermis. It demonstrates that onion epidermis is the good alternatives to rice leaf sheath to be used as plant-cell model for this study due to its hydrophobicity and penetrable surface.

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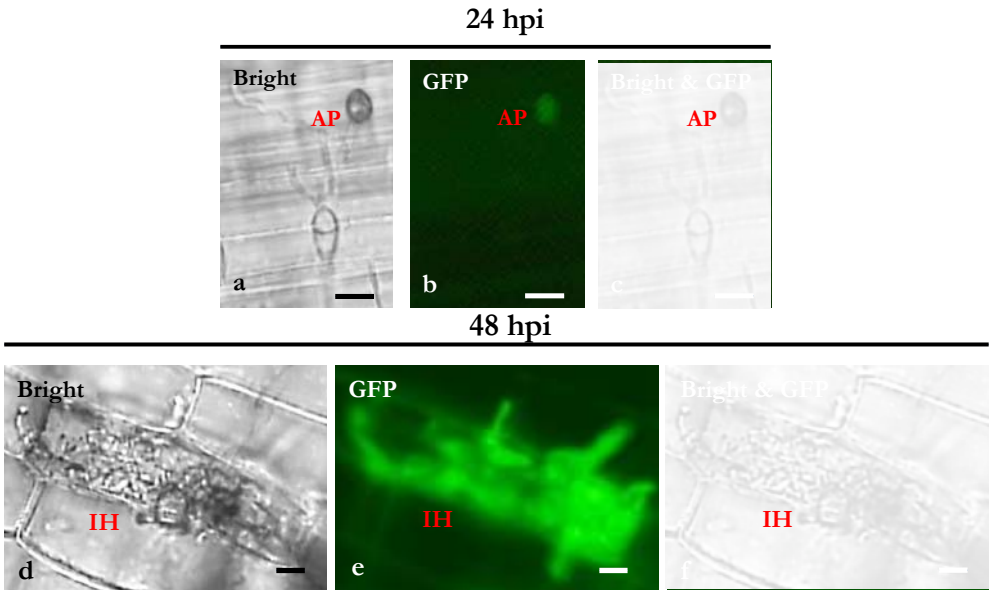

Fig. 2. Fluoresecence expression from Ina168m95-1-*PPR*::*eGFP*, inoculated on rice sheath of *Shin-2* rice at 24 and 48 hpi. Appressorium was differentiated completely (a) and *GFP* expression was clearly seen in appressoria after 24 hpi (b, c). Invasive hypha was developed inside rice cell (d), and fluorescent signal was found in all invasive hypha (e, f). Microscopic observations were performed at ×20 magnification under bright light (a, d) and under blue light (b, e). AP, appressorium; IH, invasive hypha; Bar = 10 μm.

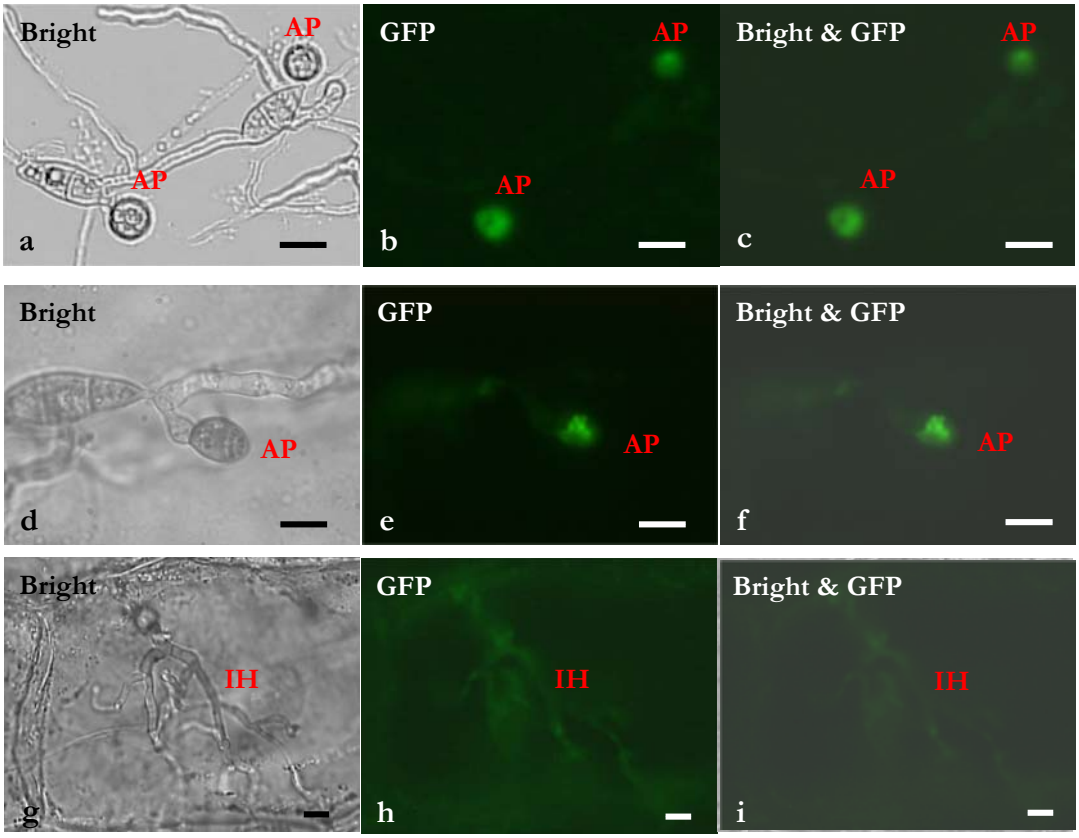

Fig. 3. *AVR-Pia* expression on glass slide and onion epidermis. *M. oryzae* Ina168m95-1 transformants carrying the *PPR*::*eGFP* were inoculated on glass slide (a) and onion epidermis (d, g) 24 hpi. Well-melanized appressoria were formed on glass slide (a) and onion epidermis (d). Fluorescence was seen in appressoria on glass slide (b) and onion epidermis (e). Inside onion cells, invasive hypha were developed (g) and *GFP* expression was induced in invasive hypha (h, i). Microscopic observations were performed at ×20 magnification under bright light (a, g) and under blue light (b, h), and at ×100 magnification under bright light (d) and blue light (e). AP, appressorium; IH, invasive hypha; Bar = 10 μm.

4. Discussion

According to the ability of avirulence effectors to control rice blast disease via recognition of AVR protein and specific R protein, study of avirulence effectors is important to understand such relationship between fungal pathogens and host plants in order to find the best way to protect our crops. Many experiments on avirulence gene of *M. oryzae* have been done on the topic of characterization, protein-protein interaction, and gene mutation. We cannot say that those topics are not important information to be known about avirulence effectors, but another topic of avirulence gene is also necessary, that is gene expression. Understanding of gene expression becomes an important tool for almost topics that related to molecular biology of living organisms. Gene expression has been known as 'The central dogma' of molecular biology that explains the flow of genetic information from DNA to RNA via transcription and then from RNA to protein via translation. Therefore, study of gene expression provides us to understand how to regulate protein production, including any factors that effect gene expression. As mentioned that avirulence effectors are related to plant immunity induction, thus if we can understand gene regulation of avirulence effectors, it should be possible that we can control rice blast disease via avirulence effectors.

In this study, we monitored *AVR-Pia* expression during fungal development and host plant infection using a powerful reporter gene, fluorescence protein (*GFP*) gene, and find the alternative cell-membrane model for *AVR-Pia* expression analysis. AVR-Pia effector was first discovered from sequencing of genome of *Ina168*, an isolate that contain nine AVR genes. AVR-Pia is a small protein, comprising 85 amino acids, while other AVR proteins are larger such as AVR-Pita (223 amino acids) and PWL (138 to 147 amino acids). Overexpression of *AVR-Pia* gene indicates that AVR-Pia is recognized by Pia resistant protein [10]. *AVR-Pia* sequence was identified as 255 bp in length including 57 bp of secretion signal [8]. Moreover, the 447 bp of sequence prior to *AVR-Pia* was clarified as the putative promoter region of *AVR-Pia* (*PPR*). In order to study *AVR-Pia* expression of *M. oryzae* during infection, *eGFP* gene was used to follow the gene expression. To construct an expression vector, only native promoter of *AVR-Pia* or *PPR* was attached to *eGFP* gene. The 255 bp of *AVR-Pia* was not included in the expression vector because the signal peptide will lead AVR-Pia protein to localize in final destination after translation and it may interfere the evaluation of the correct timing of expression. The fragment of *PPR*::*eGFP* was constructed and ligated to the vector pCSN43-DEST, containing *hygromycinB* resistant gene. The expression vector of *PPR*::*eGFP* was introduced into *M. oryzae* mutant *Ina168m95-1* (*Δavr-Pia*). Fluorescence observation was done using intact leaf sheath assay. Fluorescence was seen in appressoria on leaf sheaths of *Shin-2* (*Pia*) rice and invasive hypha, which fully colonized in rice cell after 48 hpi. It suggests that *AVR-Pia* expression is induced in appressoria and invasive hypha of *M. oryzae* by *PPR* and it may be possible that *AVR-Pia* expression is related to appressorial differentiation and fungal infection. However, further experiments are needed to clarify that hypothesis. *ACE1* gene expression has been reported that the expression is connected to the onset of appressorial-mediated penetration using a transcriptional fusion between *ACE1* promoter and *eGFP* [12], therefore, this EGFP reporter method might be applicable for the expression monitoring other AVR genes in *M. oryzae*.

Rice leaf sheath assay is a most reliable method for the observation of *M. oryzae in planta* growth, but is also a time-consuming method. It takes a couple of months to prepare rice cultivars. In this study, we would like to shorten the time by using other surfaces or membranes. Previously, artificial membranes were used to study surface attachment in spores of *M. oryzae*. It was reported that conidia germinated and formed germ tubes but no appressoria formation on glass surface, while on Teflon-PFA film, conidial germination and appressorial formation were found [25]. It was said that artificial surfaces that can induce appressorium differentiation might have properties same as rice surface. However, artificial membranes absolutely cannot be penetrated by fungal pathogen. Therefore, it limits the study of fungal pathogen-host interaction, including infection. In this study, we examined the function of onion epidermis to be used as infection assay to study expression of *AVR-Pia*. The transformant Ina168m95-1-*PPR*::*eGFP* was inoculated on heat-killed onion epidermis, and observed fluorescence. The results showed that well-melanized appressoria were differentiated, and colonization by invasive hypha was seen on onion epidermis. Moreover, the *eGFP* gene was expressed in appressoria and invasive hypha on onion cells at 24 hpi, similarly to the results from rice sheath assay. It suggests that we can use onion epidermis instead of rice sheath to study *AVR-Pia* expression, and may include other avirulence effector genes. In addition, we also found that appressoria were normally developed on glass slide after 24 hpi, contrast with the results from previous report [25]. Fluorescence was also seen clearly in appressoria on glass slide. It demonstrates that glass slide is one of

artificial membranes that *M. oryzae* can differentiate appressoria and can express *AVR-Pia* gene in appressoria, and that no plant-derived materials are required for the induction of *AVR-Pia* gene. Therefore, this study revealed that glass slide assay is available to study fungal gene expression in appressorium.

Overall, these experiments demonstrate that the expression vector, containing promoter of *AVR-Pia* (*PPR*) and *eGFP*, is function for studying of *AVR-Pia* expression. In addition, onion epidermis is an alternative membrane to study fungal gene expression during infection. We expect that this study will be beneficial for studying molecular biology of plant-fungal interaction.

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